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Hepatic Metabolism of the Environmental Mutagen 2-Nitrofluoranthene

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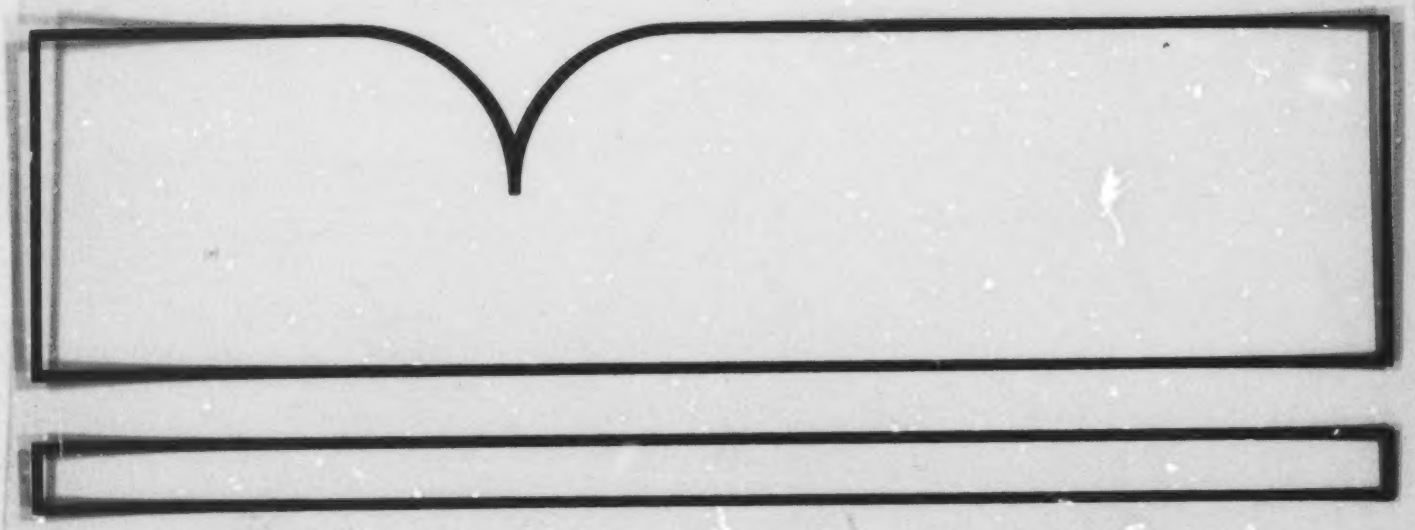

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HEPATIC METABOLISM OF THE ENVIRONMENTAL MUTAGEN 2-NITROFLUORANTHENE

By

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16. ABSTRACT

2-Nitrofluoranthene (2-NFA, Fig. 1) has recently been demonstrated to be the most abundant nitrated fluoranthene in organic extracts of ambient air particulate matter collected both in Philadelphia (Nishioka et al., unpublished results) and in Southern California (1). Whereas the better-known isomer 3-nitrofluoranthene (3-NFA) is found in combustion emissions such as diesel exhaust (2) and arises from direct nitration by NO_2/N_2O_4 or the NO^+_2 ion, 2-NFA appears to be formed from fluoranthene in the ambient air by a specific atmospheric transformation mechanism, possibly initiated by addition of an OH radical at the site of highest electron density (Fig. 2). An analogous pathway would lead to the formation of 2-nitropyrene which was observed in rural ambient air particulate matter in Denmark (3).

Other nitrated polycyclic aromatic hydrocarbons (nitroPAH) present in the environment are known to be potent bacterial mutagens (4). Their genotoxicity in prokaryotes is largely due to generation of active electrophiles [leading to formation of covalenty-bound C8-deoxyguanosine DNA adducts (5)] by nitroreduction. In vivo and in vitro studies on the routes of metabolism and mechanisms of activation of 1-nitropyrene, a model nitroPAH, have attempted to elucidate the extent to which this class of compounds represents a potential health risk to mammalian organisms (6,7,8,9,10,11).

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HEPATIC METABOLISM OF THE ENVIRONMENTAL MUTAGEN 2-NITROFLUORANTHENE

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INTRODUCTION

2-Nitrofluoranthene (2-NFA, Fig. 1) has recently been demonstrated to be the most abundant nitrated fluoranthene in organic extracts of ambient air particulate matter collected both in Philadelphia (Nishioka et al., unpublished results) and in Southern California (1). Whereas the better-known isomer 3-nitrofluoranthene (3-NFA) is found in combustion emissions such as diesel exhaust (2) and arises from direct nitration by NO_2/N_2O_4 or the NO^{+2} ion, 2-NFA appears to be formed from fluoranthene in the ambient air by a specific atmospheric transformation mechanism, possibly initiated by addition of an OH radical at the site of highest electron density (Fig. 2). An analogous pathway would lead to the formation of 2-nitropyrene which was observed in rural ambient air particulate matter in Denmark (3).

Other nitrated polycyclic aromatic hydrocarbons (nitroPAH) present in the environment are known to be potent bacterial mutagens (4). Their genotoxicity in prokaryotes is largely due to generation of active electrophiles [leading to formation of covalenty-bound C8-deoxyguanosine DNA adducts (5)] by nitroreduction. *In vivo* and *in vitro* studies on the routes of metabolism and mechanisms of activation of 1-nitropyrene, a model nitroPAH, have attempted to elucidate the extent to which this class of compounds represents a potential health risk to mammalian organisms (6,7,8,9,10,11).

We report here the identification of some of the metabolites formed from 2-NFA by Aroclor-treated rat liver S9.

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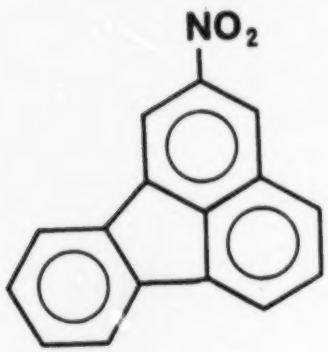

Figure 1. Structure of 2-nitrofluoranthene

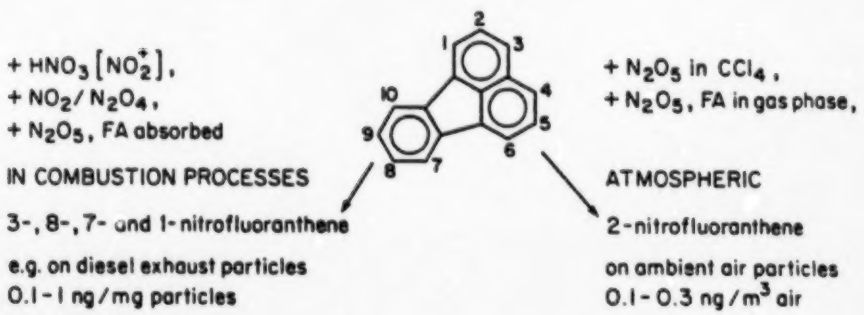

Figure 2. Products of nitration of fluoranthene

MATERIALS AND METHODS

Chemicals

2-Nitrofluroanthene (2-NFA; CAS #13177-29-2) was synthesized from 3-aminofluoranthene (Aldrich Chemical Co., Milwaukee, WI) as described (12). The product was chromatographed on open-bed silica gel (eluted with benzene) and recrystallized from glacial acetic acid until yellow needles were obtained with m. p. 149.5 - 150°C, uncorr. (lit. 153 - 153.5°C, [12]). The purity of the product was verified by HPLC and TLC. Other chemicals and solvents were purchased from commerical suppliers, mainly Fisher Scientific, Raleigh, NC.

Incubation of 2-nitrofluoranthene with S9

2-NFA (final concentration 150 μM) dissolved in acetone was added to Aroclor-treated rat liver S9 fraction (1 mg protein/mL in 0.1M HEPES buffer, pH 7.4; final vol. 80 or 133 mL) supplemented with an NADPH-generating co-factor mix consisting of $MgCl_2$, 8 mM, KCl, 33 mM, glucose-6-phosphate, 5 mM and NADP, 4 mM. The reaction was terminated (after 1 h at 37°C) by addition of 1 vol. of ice-cold ethyl acetate/acetone (2:1, v/v). The reaction products were extracted with a further 2 x 1 vol. of ethyl acetate/acetone. The pooled organic extracts were concentrated (to approx. 3 mL) by rotary evaporation, then taken to dryness under a stream of N_2 gas. The residue was dissolved in MeOH (1 mL) for analysis. Control incubations were conducted in parallel, with acetone but no 2-NFA.

Isolation and analysis of 2-nitrofluoranthene metabolites

The profile of organic-extractable metabolites was determined by HPLC with a gradient from 75 to 100% MeOH in H_2O (over 15 min); fractions were collected according to the UV (254 nm) absorbance of the eluate.

Instrumentation

Proton NMR spectra were recorded in acetone-d_6 on a Brucker WM-250 instrument. Mass spectra were obtained by HRGC/NCI-MS on a Finnegan 2500 gas chromatograph/EI-CI mass spectrometer (Finnegan Instrument Co., Sunnyvale, CA) operated in the NCI mode with CH_4 as the reagent gas. Melting points were determined on a Hoover capillary melting point apparatus (A.H. Thomas Co., Philadelphia, PA) and are uncorrected throughout. UV-vis spectra were recorded on a Cary 2200 spectrophotometer (Varian Techtron Pty Ltd, Mulgrave, Australia), and fluorescence spectra on

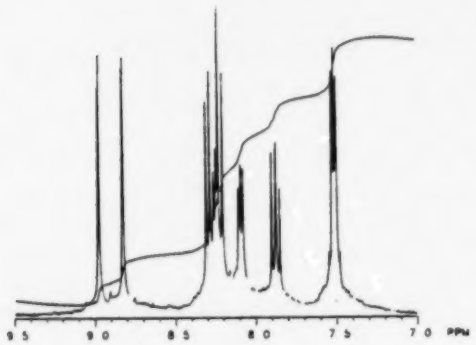

Figure 3. Proton NMR spectrum of 2-nitrofluoranthene (synthesized according to Kloetzel et al., 1955 [12]), in acetone-d_6

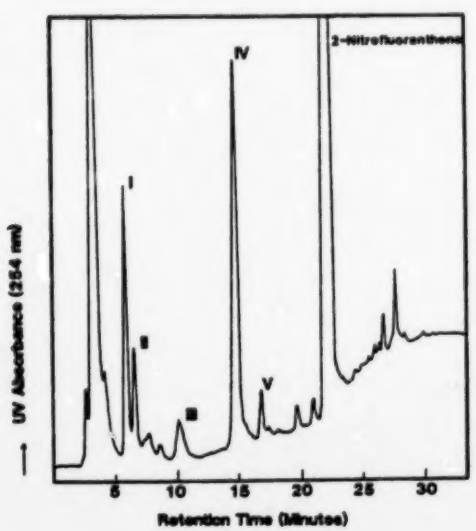

Figure 4. HPLC profile of ethyl-acetate extractable metabolites of 2-nitrofluoranthene formed by Aroclor-treated S9

an in-line Perkin-Elmer MPF-4 fluorometer (Perkin-Elmer, Norwalk, CT). HPLC was performed on a Varian 5000 (Varian Instrument Co., Palo Alto, CA) chromatograph equipped with a Zorbax ODS (4.6 x 250 mm) column (DuPont Instrument Co., Wilmington, DE) for analytical purposes. The UV absorbance was monitored at 254 nm and on-line UV spectra determined with a Perkin-Elmer LC-85B spectrophotometric detector (Perkin-Elmer, Norwalk, CT). Metabolite fractions were isolated with a Zorbax ODS 9.4 x 250 mm column.

RESULTS

2-NFA was synthesized and shown to be over 99% pure by HPLC analysis. The substitution pattern of this nitrofluoranthene isomer was confirmed by proton NMR spectroscopy (Fig. 3). The principal features of the NMR spectrum of 2-NFA are also important characteristics in elucidating the substitution pattern of 2-NFA metabolites. The protons adjacent to the nitro group are prominent downfield singlets with meta coupling detectable ($J \simeq 1.5$ Hz). H_1 (δ = 8.98 ppm) is located downfield of H_3 (δ = 8.83 ppm) on account of its situation, projecting into a pseudo-bay region. The protons on the b ring of the naphthalene moiety give rise to a distinctive pattern: a triplet (H_5, δ = 7.87 ppm) coupled to H_4 (8.22 ppm; 8 Hz) and H_6 (8.30 ppm; J = 7 Hz). The distal protons H_8 and H_9 on the benzo ring appear as two overlapping triplets, 7.52-7.49 ppm, coupled to each other and to H_{10} (8.24 ppm) and H_7 (8.08 ppm) respectively ($J \simeq 6$ Hz). The signal due to H_{10} is shifted slightly downfield from that due to H_7 because of the long-range deshielding effect of the nitro group.

2-NFA was incubated with Aroclor-treated rat liver S9 for 1 h at 37°C. Five major peaks (I - V) and unchanged parent compound were detectable on HPLC analysis of the concentrated extracted reaction media (Fig. 4). Measurement of the area of the chromatographic peak corresponding to unchanged 2-NFA indicated that 33 to 50% of the substrate had been consumed during the incubations. This corresponds to substrate disappearance rates up to 75 nmoles/mg protein in 1 h. The individual product peaks I - V were collected separately from the HPLC eluates, rechromatographed where necessary, then analyzed by two or more of UV-visible spectroscopy, proton NMR spectroscopy, and HRGC/NCI mass spectrometry.

The most polar peak (I) contained two components, one major component which dominates the proton NMR spectrum of this peak (Fig. 5), and one minor component. The minor

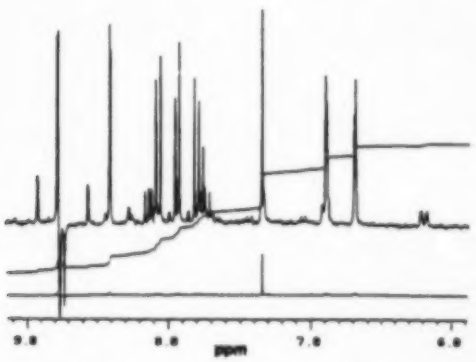

Figure 5. Proton NMR spectrum of metabolite I formed from 2-nitrofluoranthene (in acetone-d_6)

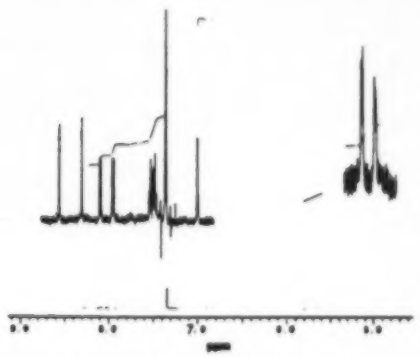

Figure 6. Proton NMR spectrum of metabolite II fromed from 2-nitrofluoranthene (in acetone-d_6)

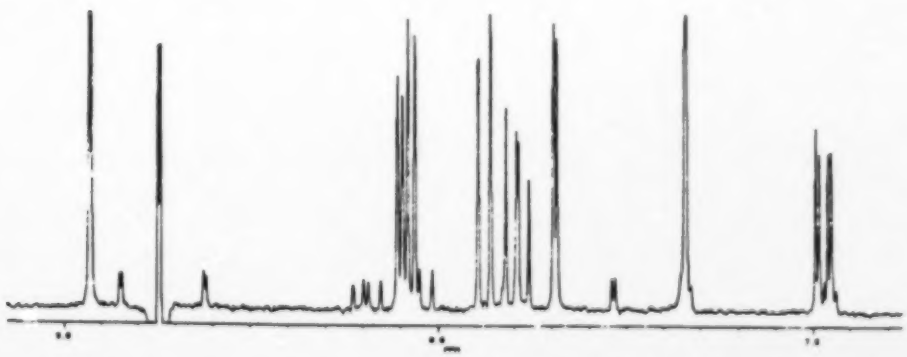

Figure 7. Proton NMR spectrum of 2-nitrofluoranthene metabolites IVa (minor) and IVb (major component) in acetone-d_6

component showed resonances attributable to H_1 and H_3 (8.9 and 8.8 ppm, respectively), and a pair of doublets at 6.20 and 6.17 ppm; this component can be inferred to be a dihydrodiol, but insufficient quantities were present for further purification and definitive characterization.

The proton NMR spectrum of the major component in peak I (Fig. 5) contained signals clearly attributable to H_1 and H_3 (8.78 and 8.41 ppm respectively), and the triplet H_5 (7.79 ppm) coupled to H_4 (8.07 ppm) and H_6 (7.94 ppm), indicating that the naphthalene rings were intact. Two quasi-singlets with fine splitting corresponding to meta or para coupling (2.0 Hz) were assigned to H_7 and H_{10} (6.69 and 6.89 ppm, respectively), shifted upfield relative to their positions in the parent 2-NFA (Fig. 3). No signals were found which could be attributed to other protons on the distal benzo ring. The upfield shift of H_7 and H_{10} indicated that both were in proximity of one or more hydroxyl groups; the absence of benzylic protons signifies that the substitution took the form of a catechol (or di-quinone) rather than a dihydrodiol.

Peak II appeared as a single component on NMR analysis, with a spectrum (Fig. 6) in which protons H_1 and H_3 (8.55 and 8.29 ppm), and the protons on the distal benzo ring could clearly be distinguished (δ 8.08, d, J=7.6 Hz, H_{10}; δ 7.93, dd, J=6.8, 1.5 Hz, H_7; 7.54, m, J=1.6, 7.0, 7.3 Hz, H_8; 7.48 m, J=1.8, 7.0, 7.6 Hz, H_9). The remaining unassigned signals were those of a pair of benzylic proton, one (at 5.14 ppm) cleanly split (J=7.5 Hz), the other at 4.97 ppm multiply split (J=7.5 and 3.0 Hz) and also coupled to a doublet (δ=6.99 ppm, J=2.4 Hz). Nuclear Overhouser irradiation of the signal corresponding to H_3 resulted in a two-fold enhancement of the intensity of the peak at 6.99 ppm, indicating its close physical proximity to H_3. This signal can therefore be attributed to H_4, thus the peaks at 4.97 and 5.14 ppm correspond to benzylic protons on H_5 and H_6 respectively; this metabolite fraction can therefore be unambigously identified as 2-nitrofluoranthene-5,6-dihydro-5,6-diol. The coupling between between H_5 and H_6 (7.5 Hz) is consistent with a <u>trans</u> configuration of the dihydrodiol.

Peak III, although close in retention time to 2-aminofluoranthene, was not identifiable as such, and appeared to disappear on work-up.

Peak IV when analyzed by HRGC/MS with negative chemical ionization was separated into two clearly distinct (although not entirely resolved) major components, present in similar quantities, each of which had m/z 263 and loss

of 16 mass units characteristic of a monohydroxylated species. Rechromatography on reverse phased HPLC with a more shallow gradient than that used for the initial analysis resulted in separation of two distinct peaks IVa and IVb; the UV-visible spectra of both these exhibited the reversible bathochromic shift (in alkali) characteristic of phenols. These closely-related species were therefore clearly nitrofluoranthenol isomers. Assignment of their positions of hydroxylation was possible by comparison of their proton N.M.R. spectra. Fig. 7 shows a spectrum determined on an incompletely-resolved sample in which metabolite IVb was the major and metabolite IVa was the minor component. In both cases the meta-split singlets adjacent to the nitro group (H_1 and H_3) and the triplet characteristic of H_5 (coupled to the doublets H_4 and H_6) can be recognized, indicating that no changes have occurred on the naphthalene rings. The pattern of the remaining signals is an upfield meta-coupled singlet and an a b quartet corresponding to a pair of protons ortho to each other, one of which is shifted upfield, and also shows some further fine splitting arising from meta coupling. The upfield shift is due to proximity to a hydroxyl group, therefore these closely-related compounds can be identified as 2-nitrofluoranthen-8- and -9-ol. Since in the spectrum of the parent 2-NFA H_{10} is 0.16 ppm downfield from H_7 (due to the long-range deshielding effect of the nitro group), then similarly the singlet signal due to H_{10} in 2-nitrofluoranthen-9-ol is predicted to lie downfield of the singlet due to H_7 in 2-nitrofluoranthen-8-ol. Therefore the spectrum containing the most down-field-shifted singlet (δ=7.69) corresponds to 2-nitrofluoranthen-9-ol (metabolite IVa) and metabolite IVa is 2-nitrofluoranthen-8-ol.

Peak V was shown by HRGC/MS to be also a nitrofluoranthenol, but was isolated in quantities low to permit conclusive isomer identification.

DISCUSSION

2-NFA undergoes extensive oxidative metabolism, predominantly on the benzo ring distal to the nitro group. The identification of the major metabolites of 2-NFA as 2-nitrofluoranthen-8- and -9-ol is in accord with the observations of Zielinska et al. (13); the previously unreported 5,6-dihydrodiol argues for epoxidation as an initial step in the oxidation of the naphthalene b ring. The detection of both 8- and 9-hydroxy isomers in similar quantitities suggests either initial epoxidation across the C8-C9 bond, followed by symmetric rearrangement to

both phenols, or direct hydroxylation with no positional preference. The absence of any identifiable 2-nitrofluoranthen-8,9-dihydro-8,9-diol, and the tentative identification of Metabolite I as an 8,9-catechol or quinone [which could arise from decomposition of the catechol, as was observed for dibenzo(a,e)fluoranthene (14)], lends support to the second hypothesis, but this evidence is not conclusive at present, since the first ten minutes of the HPLC elution profile may contain more minor metabolites as yet undetected. Quantitative studies with the epoxide hydrase inhibitor 3,3,3-trichloropropylene oxide may help resolve this question.

No oxidation was observed at carbons 1 and 3; these positions would be not only deactivated towards electrophilic substitution but also sterically hindered by the presence of the nitro group at position 2. Unlike with 1-nitropyrene under similar conditions (7), but as also with 3 NFA (15), no products of nitroreduction were observed. This suggests that the nitrofluoranthenes may generally be less susceptible to reduction by mammalian reductase enzymes in an aerated environment. Epoxidation of the aromatic rings (implicit in the identification of 2-nitrofluoranthen-5,6-dihydro-5,6-diol, and possibly also occurring across the C_8-C_9 bond) would itself generate a potentially reactive electrophile by opening of the oxirane ring, capable of interacting with cellular nucleophiles such as protein and DNA bases. This eventually would provide a pathway for genotoxic activation of 2-NFA by solely mammalian enzymic pathways, independently of any nitrenium ion generation via nitroreduction.

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